Quantitative Selection of Respiratory Deficient Mutants in Yeasts by Triphenyltetrazolium Chloride

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(Z. Naturforsch. 27 b, 252—256 [1972]; received January 14, 1972)

Dedicated to Prof. WERNER SCHÄFER on the occasion of his 60th birthday

Triphenyltetrazolium chloride exhibits a strong growth inhibition in respiratory competent cells but shows only minor effects in respiratory deficient mutants of Saccharomyces cerevisiae and Schizosaccharomyces pombe. Use of this dye thus allows rapid selection of rarely occurring respiratory deficient mutants, showing karyotic as well as extrakaryotic inheritance. Mutation induction by tetrazolium chloride was not observed. The results favour the hypothesis that triphenyltetrazolium chloride interferes with the terminal oxidase of the respiratory chain.

Materials and Methods

Strains

Schizosaccharomyces pombe:
972 h; 50/1 a h+ ade 7; 50 h- ade 7. These strains were kindly provided by Prof. Dr. U. LEUPOLD, Berne.

Saccharomyces cerevisiae:
M 12 a ile 5, try 2, ura 3. A 1328 A a, ade 2, leu 1 (Seattle Yeast Stock Culture).

Media

Schizosaccharomyces pombe:
Glucose Medium: 1 per cent yeast extract (Merck), 2 per cent (w/v) glucose (unless otherwise stated). Glycerol Medium: 1 per cent yeast extract, 2 per cent (v/v) glycerol.

TTC Medium: 2,3,5-triphenyltetrazolium chloride (TTC) (Merck) added as a sterile solution to the autoclaved glucose medium.

Co Medium: cobalt sulphate (Merck) added to glucose medium.

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TTC media must be stored in the dark. Media were solidified with 1.5 per cent agar and supplemented with adenine (100 mg/ml) if necessary.

Saccharomyces cerevisiae:
Media as for Schizosaccharomyces, enriched by 2 per cent peptone (Merck).

Cultures
Cells used for all experiments were grown to early stationary phase. They were obtained after inoculation of liquid glucose medium with about 10\(^6\) cells/ml and incubation at 30 °C with aeration for 24 hours (Saccharomyces) or 36 hours (Schizosaccharomyces).

Results

1) Selective growth inhibition by TTC

a) Saccharomyces cerevisiae

Differential staining of respiratory competent (RC) and respiratory deficient (RD) colonies with 2,3,5-triphenyltetrazolium chloride has widely been used as a diagnostic means. TTC, when added to glucose agar plates (50 µg/ml) or to overlay agar (1 mg/ml), stains RC colonies pink to red but leaves RD colonies white\(^3\)\(^4\). In our experiments TTC concentrations of 10 to 30 µg/ml agar led to similar results. With increasing TTC concentrations in glucose agar (100, 150, 200 µg/ml), selective inhibition of RC colonies is observed whereas RD colonies show only minor reduction in colony size (Table 1).

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<table>
<thead>
<tr>
<th>TTC µg/ml</th>
<th>No. of cells plated</th>
<th>No. of colonies grown*</th>
<th>No. of RD colonies*</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>170</td>
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<tr>
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</tbody>
</table>
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Table 1. TTC-tolerance of RD and RC cells of strain A 1327 A. *The average value of 5 plates. Respiratory deficiency was proved by replica plating on glycerol agar.

Under these conditions rare petite mutants were easily selected by plating 10\(^4\) to 10\(^5\) cells per plate. After 4 to 6 days of growth at 30 °C, RD cells reached normal colony size. In a relevant experiment the following results were obtained after plating 10\(^4\) liquid grown cells of strain M 12 on glucose medium with 150 µg/ml TTC:

1. Colonies of normal size which, after replica plating, were not able to grow on glycerol medium. These were isolated as respiratory deficient cells. Their number differed from 5 to 6 per plate.
2. Barely visible, deep red coloured micro-colonies which were able to grow on glycerol medium. Their number (about 20 to 40 per plate) varied and because of their small size it was difficult to score them.
3. Rare colonies of a size between those of 1 and 2 showed red colour and were able to grow on glycerol medium. Their ability to grow on TTC medium was not permanent and after a passage on glucose medium their growth on TTC was sometimes blocked in the same way as that of wild type cells.

The inability of the colonies of type 1 to use glycerol as an energy source remained a constant property. These RD mutants were crossed to RC cells and shown to be extrakaryotic (cytoplasmic) petites. Their frequency in strain M 12 was about 4·10\(^{-4}\) as was revealed from TTC plates. This is in good agreement with the data reported by Schwaier et al.\(^17\) who found a frequency of 5.5·10\(^{-4}\) for the spontaneous mutation rate of RD mutants. The same effect of TTC was observed with cells of strain A 1327 A which shows a high mutation rate to RD. From 200 cells plated on TTC medium about 70 colonies were visible after 4 days of incubation; more than 90% of them proved to be respiratory deficient in a control experiment on glucose medium without TTC, 50 to 70 colonies out of 200 were unable to grow on glycerol medium. Increase of TTC concentration to 250 µg/ml resulted in a reduction of the number and size of RD colonies after 6 days of growth. The colour of petite colonies changed to pink and red. With doubled TTC concentration (500 µg/ml), no cells were able to produce colonies. When the TTC concentration was decreased to 30 µg/ml, a dense lawn of rose coloured colonies developed 3 days after plating 10\(^4\) cells/plate of strain M 12. White RD colonies were only slightly larger and their detection was difficult.

b) Schizosaccharomyces pombe, selective growth on solid media

Lower TTC concentrations proved to be optimal for selection of RD mutants in Schizosaccharomyces. Comparable to the experiments with Saccharomyces, 3 classes of colonies were obtained on glucose agar.
with 30 mg TTC per liter: 2 to 3 colonies of type 1 (large, white colonies, RD), 100 to 150 colonies of type 2 (very small, dark red colonies, RC) and 2 to 3 colonies of type 3 (medium size, red coloured, RC), each per 10^5 cells plated.

In a control experiment, respiratory competent cells (ade 7), auxotrophic for adenine, were mixed in a ratio of about 10 to 1 with respiratory deficient cells of the same mating type, which were prototrophic for adenine. On the average 400 cells/plate were plated on TTC agar. After 6 days about 40 large, nearly white RD colonies were observed per plate, none of which was ade^-1. During prolonged incubation about 5 to 8 small red coloured RC colonies per plate developed which were ade^-1 with few exceptions. After plating cells from the same mixture on glucose agar and after replica plating on glycerol medium, the same percentage of RD mutants was found. Glucose concentrations of 2 to 3% were optimal. In solid media with 1% glucose the RD mutants no longer were able to form colonies of normal size. Incubation times lasting more than 10 days turned out to be of no advantage because the small red RC colonies grew larger; after another 10 days they appeared as large, pink colonies with a red area in the center only.

c) Schizosaccharomyces pombe, enrichment of RD mutants in liquid media

The selective growth inhibition of RC cells by TTC might be expected to lead to an enrichment of RD cells in a mixed population growing in liquid culture in the presence of TTC. In the experiment described in Fig. 1 a mixture of RD and RC cells (about 5 per cent RD, ade^+ and 95 per cent RC, ade^-7, cells, both with mating type h^-) was grown under the following conditions:

1. In a medium containing 0.5 per cent glucose and varying TTC concentrations, static culture without aeration,
2. In a medium containing 10 per cent glucose and varying TTC concentrations, static culture without aeration,
3. In a medium containing 0.5 per cent glucose, varying TTC concentrations and constant rapid aeration.

Early stationary phase cultures (6 to 8 generations in 1 and 3, 9 to 10 generations in 2) were plated on glucose agar. After replica plating on glycerol agar the percentage of RD colonies was determined. With optimal TTC concentrations (50 to 60 mg per litre) the percentage of RD cells increases from about 5 to 75 per cent (Exp. 1). Both high glucose concentrations and aeration nearly eliminate the effect of TTC in liquid cultures (Exp. 2 and 3). Since all RD colonies were adenine prototrophs, this increase of RD cells was due to enrichment of preexisting mutants and not to mutation induction in RC cells requiring adenine.

A possible enrichment of RD cells caused by inactivation of RC cells could be excluded. In an experiment with an RC strain, the percentage of RC cells forming colonies on glucose agar after growth in TTC concentrations from 0 to 70 mg per litre was nearly constant in all cases. This implies that enrichment of RD cells in mixed populations is due to growth inhibition not to inactivation of RC cells. The degree of enrichment depends on the number of cell generations. In the case of a lower percentage of RD cells as described above, a prolonged growth in TTC medium is required for an efficient enrichment.
II) Characterization of mutants selected by TTC

Both extrakaryotic and karyotic mutants were selected in Saccharomyces cerevisiae. Enrichment of karyotic mutants was proved with cells of the mutant pet-6 and pet-7, which complement to rho- "petite" cells. In Schizosaccharomyces pombe, genetic analysis of about 50 spontaneous and UV-induced RD mutants selected by TTC revealed that all mutants showed Mendelian inheritance of respiratory deficiency. From the fact that we failed to find extrakaryotic mutants after isolation by replica plating (30 mutants tested), it may be assumed that this type of RD mutants if present, is rare in Schizosaccharomyces.

Biochemical characterization of TTC selected mutants proved that a wide range of mutations lead to a growth advantage in the presence of TTC. These include mutations affecting complex I, II, III and IV of the respiratory chain as was shown by studies on respiratory enzymes and cytochromes 8, 18.

III) Selection of RD mutants by CoSO₄

HORN and WILKIE 15 reported that in a cobalt medium cytoplasmic respiratory deficient mutants of Saccharomyces cerevisiae show a selective advantage.

Although we too found selective growth of respiratory deficient cells in cobalt medium, the results were not as clearcut as in experiments with TTC. Depending on yeast strain and culture conditions, the selective advantage of RD cells was variable.

In Schizosaccharomyces pombe RD cells also exhibit a selective advantage in cobalt media. In the experiment shown in Table 2, a mixed population of karyotic RD cells and wild type cells was plated on agar containing varying CoSO₄ concentrations. With increasing concentrations wild type colonies are inhibited to a higher degree than RD cells. As with TTC, a growth advantage is also observed in liquid cobalt media. Rapid aeration diminishes the effect. Since only karyotic RD mutants have so far been found in Schizosaccharomyces, the observation made by HORN and WILKIE 15 that the selective advantage is to be restricted to extrakaryotic RD mutants (rho-) is not generally true.

<table>
<thead>
<tr>
<th>CoSO₄ concentration</th>
<th>No. of cells plated</th>
<th>No. of colonies grown per plate</th>
<th>No. of RD cells per plate</th>
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Table 2. CoSO₄ tolerance of RD and RC cells in Schizosaccharomyces pombe. A mixture of RC and RD (karyotic) cells was plated on glucose agar containing varying CoSO₄ concentrations. The plates were incubated at 30 °C for 5 days. Respiratory deficiency was proved by replica plating.

Discussion

The results presented above demonstrate that selective growth of RD colonies is achieved by plating a mixed population of RD and RC cells on agar containing triphenyltetrazolium chloride. The RD colonies arising on TTC agar are not due to mutations induced by the dye but grow from preexisting mutant cells. This is shown by two experiments:

A) The percentage of RD cells revealed by plating a cell population on TTC agar was not increased compared to that revealed by plating on glucose agar and by replica plating on glycerol.

B) A mixture of auxotrophic RC and prototrophic RD cells was grown in the presence of TTC. All RD mutants selected by the dye were found to be prototrophic, i.e. no mutation to respiratory deficiency had occurred in RC cells.

C) Neither was there any mutation induced with TTC concentrations lower than those used for selection.

W. LASKOWSKI 14 reported a mutagenic effect for TTC on a strain of Saccharomyces. A concentration higher than 1·10⁻⁴ induced 100 per cent "petite" mutants. This incompatibility of results has not yet been explained. Our experiments demonstrate the selective effect of TTC to be due to growth inhibition of respiring cells. A decrease in respiratory activity due to high glucose concentrations reduces the selective effect of TTC. There was no inactivation of cells, even with highest concentrations of the dye.
Cobalt sulphate, which was reported to select extrakaryotic RD mutants in Saccharomyces cerevisiae, also leads to a growth advantage of karyotic RD cells in Schizosaccharomyces pombe. However, the absence of physiological adaption of RC cells to TTC, the low rate of TTC-resistant mutants, as well as the wide range of concentrations allowing selective enrichment of RD cells so far render TTC to be the most efficient means for rapid selection of rarely occurring "petite" mutants.

After rapid aeration no enrichment of RD cells by TTC in liquid culture was ascertained. This is in agreement with the results obtained by SLATER et al. 19, who noticed that the succinate: TTC reductase system was blocked by rapid shaking of the suspension. This inhibition is thought to be due to the fact that the reduction of TTC to formazan in the electron pathway competes with the reduction of oxygen 19, 20. In addition to this interaction with the electron transport system, an uncoupling effect of tetrazolium salts on the oxidative phosphorylation has been described 21.

The electron potential (E'0 = 460 mv) reported for TTC 22 as well as the fact that CN− completely blocks the succinate:TTC reductase favour the hypothesis that TTC couples with the respiratory chain via cytochrome oxidase 19, 20. Further support comes from the analysis of RD mutants selected by TTC. In Schizosaccharomyces, mutants showing defects in different parts of the respiratory chain, especially in complex IV, have been isolated 9, 18. The genetic block in the electron transport chain leads to a decrease in the reduction of TTC to formazan, as can be seen from reduced colour production of the RD colonies, and thereby allows these cells to grow faster than RC cells. No share of the uncoupling effect of TTC was to be noticed in the selection of RD cells by the dye.

This investigation was supported by the Deutsche Forschungsgemeinschaft. We are indebted to the European Molecular Biology Organization for the award of a short-term fellowship to K. W. The skilled technical assistance of Mrs. HEINERICH and Mrs. SCHICHTEL is gratefully acknowledged.

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